Effects of Interferons on Cultured Human Melanocytes In Vitro: Interferon-Beta but Not -Alpha or -Gamma Inhibit Proliferation and All Interferons Significantly Modulate the Cell Phenotype

Konstantin Krasagakis, Claus Garbe, Sabine Krüger, and Constantin E. Orfanos
Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Berlin, Germany

The effects of human recombinant interferon-alpha-2a (rIFN-α), natural interferon-beta (nIFN-β) and recombinant interferon-gamma (rIFN-γ) on the proliferation, morphology and antigen expression of cultured human melanocytes were studied in vitro. The investigations were performed in 12-O-tetradecanoylphorbol-13-acetate (TPA)- and serum-containing melanocyte growth medium (MGM), in TPA- and serum-free complete melanocyte medium (CMM) and its mitogen reduced variant (RMM). In MGM, none of these interferons inhibited the growth of normal melanocytes at concentrations 1–10,000 international units (IU)/ml over a period of 5 d. Only nIFN-β, dose dependently, inhibited melanocyte proliferation in CMM and RMM in a 6- and 12-d assay (growth inhibition at 10,000 IU/ml; 77–80% of the controls, $p < 0.001$). In contrast, rIFN-α and rIFN-γ exerted no (RMM), or minor effects (CMM) on melanocyte proliferation (only in 12-d assays at 10,000 IU/ml: 24% and 21% of the controls respectively, $p < 0.01$). In parallel experiments performed on melanoma cells, all three interferons were potent inhibitors of proliferation in a 5-d serum-free assay (growth inhibition at 10,000 IU/ml; rIFN-α 59%, nIFN-β 78%, rIFN-γ 56%, all $p < 0.001$). In addition, nIFN-β and also rIFN-γ caused striking morphologic changes of normal melanocytes in vitro. Especially under $\geq$ 10 IU/ml rIFN-γ cytoplasmic spreading and flattening of the cultured melanocytes and their nuclei were seen, thus resembling melanoma cells in vitro.

Untreated human melanocytes grown in MGM showed high expression of the melanoma-associated antigens HMB-45 (95–100%) and K.1.2 (40–100%), whereas the progression marker A.1.43 was present only on <5% of the cells. Cultured melanocytes were 95–100% positive for histocompatibility antigen class I (HLA-I), 30–75% were positive for ICAM-1, whereas they were negative for HLA-DR.

After treatment with rIFN-α, increased expression of HLA-I antigens was found; nIFN-β and rIFN-γ decreased the labeling with HMB-45 (75–100%) and with K.1.2 (25–80%), whereby the expression of A.1.43 was found slightly increased (5–15%). The HLA class I antigens were upregulated by both nIFN-β and rIFN-γ, nIFN-β being the most potent agent. Also, both nIFN-β and rIFN-γ increased the expression of ICAM-1 (nIFN-β, 75–90%; rIFN-γ, 90–95%) and induced de novo expression of HLA-DR antigen (nIFN-β, 15–20%; rIFN-γ, 65–95%).

The decreased antiproliferative activity of rIFN-α and rIFN-γ on skin-derived normal human melanocytes is in sharp contrast to the marked growth inhibition seen on melanoma cells in vitro. This implies that malignant transformation in the melanocytic system evokes increased sensitivity to the antiproliferative action of rIFN-α and rIFN-γ in vitro. On the other hand, nIFN-β and rIFN-γ induced changes of melanocytic morphology and antigenic profile causing close similarities to cultured melanoma cell lines. *J Invest Dermatol 97:364–372, 1991*

In recent years, interferons (IFN) have been regarded as a promising and non-toxic antitumor agent when used in vivo [1–3]. However, the exact mechanisms of the antitumor activity of interferons has not yet been elucidated. Interferons are capable of inhibiting the growth of several tumors and of normal cells in vitro [4]. Furthermore, the induction of cellular differentiation and modulation of antigen expression count for the direct effects of interferons on malignant cells [4–7].

Various reports, both in vivo and in vitro, suggest that interferons may play an important role in the treatment of human melanoma

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Reprint requests to: Dr. Konstantin Krasagakis, Department of Dermatology, University Medical Center Steglitz, The Free University of Berlin, Hindenburgdamm 30, 1000 Berlin 45, Germany.

Abbreviations:
APAAP: alkaline phosphatase–anti-alkaline phosphatase
bFGF: basic fibroblast growth factor
CMM: complete melanocyte medium
DMEM: Dulbecco's modified minimal essential medium
EDTA: ethylenediamine tetraacetic acid
FACS: fluorescence-activated cell sorter
FCS: fetal calf serum
FITC: fluorescein isothiocyanate
HLA(-I)(-DR): histocompatibility antigen (class I)(-DR)
IBMX: isobutylmethylxanthine
ICAM-1: intercellular adhesion molecule-1
IFN: interferons
IU: international units
MGM: melanocyte growth medium
MoAb: monoclonal antibody(ies)
MUH: 4-methylumbelliferyl heptanoate
nIFN-β: human natural fibroblast interferon-beta
PBS: phosphate-buffered saline
rIFN-α: human recombinant interferon-alpha-2a
rIFN-γ: human recombinant interferon-gamma
RMM: reduced melanocyte medium
TPA: 12-O-tetradecanoylphorbol-13-acetate

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[8–12]. In our laboratory the effects of various interferons on human melanoma cells have been extensively investigated [13]. Interferon-alpha (IFN-α), interferon-beta (IFN-β), and interferon-gamma (IFN-γ) all inhibited the proliferation of four established melanoma cell lines. Regarding the immunophenotype, IFN-γ was the most potent inducer of changes in antigen expression, leading to de novo expression of HLA-DR and upregulation of intercellular adhesion molecule-1 (ICAM-1), molecules that play an important role in the interaction between host's immune system and tumor cells. On the other hand, these antigens have been found augmented during melanoma progression [14,15].

Investigations of the effect of IFN on skin-derived normal human melanocytes may contribute to a better understanding of the effects of IFN on human melanoma. Recently, improved methods for obtaining pure melanocyte cultures allowed to investigate the effects of interferons, especially of IFN-γ, on the antigen expression of normal melanocytes [6,16,17]. Herlyn et al [16] showed that IFN-γ induced or augmented the expression of HLA-DR antigen and decreased the expression of nevus-associated antigens, whereas, the expression of melanoma-associated antigens remained unaltered. Tsujisaki et al [17] showed that IFN-γ but not IFN-α induced the expression of a 96-kd melanoma-associated antigen on normal melanocytes, later identified as the ICAM-1 molecule [18].

In this study we investigated the influence of recombinant IFN-alpha, natural IFN-beta, and recombinant IFN-gamma on skin-derived normal human melanocytes cultured in optimal and sub-optimal mitogenic conditions in vitro and reviewed the topic. Cell proliferation was assessed with a sensitive fluorometric microassay and the immunophenotype of the cultured cells was evaluated with numerous monoclonal antibodies using alkaline phosphatase–anti-alkaline phosphatase (APAAP) techniques and fluorescence-activated cell sorter (FACS) analysis.

MATERIALS AND METHODS

Melanocyte Culture Human newborn foreskin melanocytes were cultured either by a modification of the method of Eisinger and Marco [19] or a modification of the Gilchrest et al method [20]. Epidermal cells were grown in tissue culture flasks (Falcon 75 cm^2, Becton Dickinson, Heidelberg, FRG), in 5% CO_2, at 37°C in a humidified atmosphere. Media used were: 1) melanocyte growth medium (MGM); DMEM/Ham's F12 1:1 medium with 15 mM Hepes (Gibco, BRL, Paisley, Scotland), supplemented with 10% v/v fetal calf serum (FCS) (Seromed, Berlin, FRG), 80 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St. Louis, MO), 1 nM cholera toxin (Calbiochem, San Diego, CA, USA) 0.1 mM isobutylmethylxanthine (IBMX) (Sigma) and antibiotics (Seromed). On the first subculture pure melanocyte cultures were obtained. 2) complete melanocyte medium (CMM); MCDB 153 (Seromed) with added aminoacids [21] and supplemented with following substances: 2 mM Ca^{++}, 10 μg/ml transferrin, 5 μg/ml insulin (Sigma), 0.4% whole bovine pituitary extract (Clonetics Inc., San Diego, CA, USA), 2 ng/ml bovine basic fibroblast growth factor (bFGF) (Boehringer Mannheim, FRG), 10^{-9} M cholera toxin (Calbiochem), 5×10^{-7} M hydrocortison (Serva, Heidelberg, FRG) and antibiotics. Fetal calf serum (FCS) (2%) was added the first 3 d of the primary culture (in combination with 100 μg/ml G418 [Gibco]) and the first 24 h of each subculture. Pure melanocyte cultures were obtained as could be shown by staining with HMB-45 and S-100 antibodies using the APAAP method. A detailed characterization of these cultures will be reported elsewhere (manuscript in preparation). 3) Reduced melanocyte medium (RMM) is a mitogen-reduced variant of CMM with omitted transferrin, bFGF and cholera toxin, used to demonstrate the effects of IFN in sub-optimal mitogenic conditions. For this, cultures obtained in CMM were starved 10 d in RMM. First or second passage melanocytes from various donors (where necessary designated as donor 1, donor 2, etc.) were used for the experiments with the interferons.

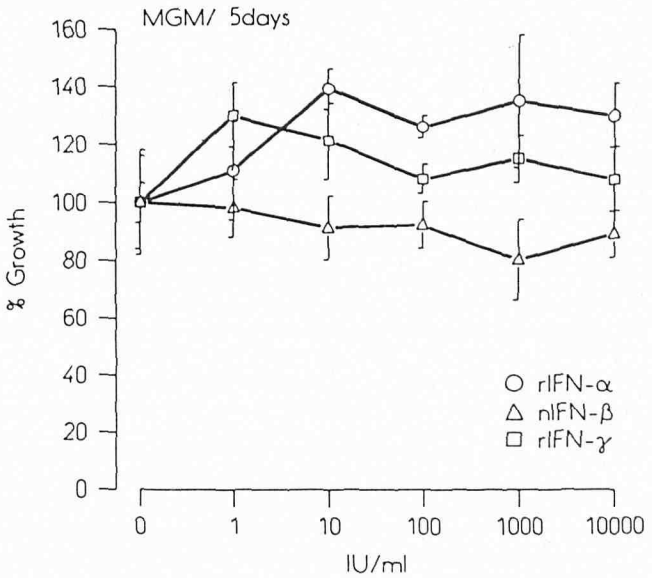

Figure 1. Proliferating foreskin melanocytes grown in MGM in five identical cultures treated 5 d with interferons: slight increases of cell proliferation appear with ≥ 10 IU/ml rIFN-α and with 1–10 IU/ml rIFN-γ ($p < 0.05$, $p < 0.01$). Representative results from one of two experiments.

Melanoma Cell Culture The established melanoma cell line SKMel-28 [22] has been used to study comparatively the effects of interferons on the proliferation of melanoma cells under serum-free conditions. Cells have been routinely maintained for 6 months in protein-free DMEM/Ham's F12 1:1. In each subculture, 10% FCS was added for 24 h to allow attachment of the cells. During interferon treatment, 1 mg/ml bovine serum albumin (Boehringer Mannheim, FRG) was added to the medium.

Interferons Human recombinant IFN-alpha-2a (rIFN-α) with a specific activity of 1.3 to 2.9×10^8 IU/mg was kindly supplied by Hoffmann-La Roche AG (FRG). Human natural fibroblast IFN-β

Table I. Specificity of Monoclonal Antibodies and Dilutions Used

MoAb	Specificity	Dilution	Source
HMB-45	anti-MAA	1:6000 (APAAP)	Enzo, NY, USA
K.1.2	anti-MAA	1:2 (APAAP)	Zelldiagnostika GmbH, Münster, FRG
A.1.43	anti-MAA	1:20 (APAAP) 1:2 (FACS)	Zelldiagnostika GmbH, Münster, FRG
L243	HLA-DR	1:40 (APAAP) 1:4 (FACS)	Becton Dickinson, Mountain View, California, USA
84H10	ICAM-1	1:400 (APAAP) 1:40 (FACS)	Immunotech SA, Marseille, France
B.9.12.1	HLA-I:45 + 12(β2m)	1:100 (APAAP) 1:10 (FACS)	Immunotech SA, Marseille, France
BL6	CD1	1:20 (FACS)	Immunotech SA, Marseille, France

(nIFN-β) with a purity of more than 80% and a specific activity 2×10^8 IU/mg was kindly supplied by Dr. Renschler AG (Laupheim, FRG). Human recombinant IFN-gamma (rIFN-γ) was kindly provided by Bioferon AG (Laupheim, FRG) and had a specific activity of $2{,}3 \times 10^7$ IU/mg protein.

Growth Experiments The proliferation assays were performed in tissue culture cluster plates (96 flat-bottomed wells) (Becton Dickinson, Heidelberg, FRG) by incubating $2-5 \times 10^3$ cells/well in 0.2 ml of various media. On day 1 after seeding cells were incubated with rIFN-α, nIFN-β, and rIFN-γ at various concentration levels (1–10,000 IU/ml). A renewal of the IFN in culture was made every 2 or 3 d together with a medium change. Cell proliferation was evaluated at times indicated in *Results* by a fluorometric microassay using 4-methylumbelliferyl heptanoate (4-MUH) (Sigma) [23,24]. Briefly, the cell monolayers were washed twice with PBS and were then incubated with 100 μl of a 100 μg/ml 4-MUH solution in phosphate-buffered saline (PBS) at 37°C. The fluorescence obtained after 30-min incubation was measured with a Titertec Fluoroscan II (Flow Laboratories, Meckenheim, FRG). The fluorescence values correlated well with cell counts performed independently. Proliferation experiments were repeated once or twice with good reproducibility.

APAAP-Immunocytochemistry For staining with monoclonal antibodies (MoAb), cytospin preparations of untreated control and IFN-treated (100–1000 IU/ml respectively) melanocyte cultures maintained in MGM were fixed 10 min in acetone and were then incubated 30 min with the appropriate MoAb dilution. In some cases, to preserve cell morphology, melanocytes were directly stained on culture substrate with the different MoAb. For this, cells were grown on glass slides using Flexiperm chambers (Heraeus, Hanau, FRG). The cells were stained with the MoAb HMB-45 as a highly specific marker for cells of melanocytic origin [25], the

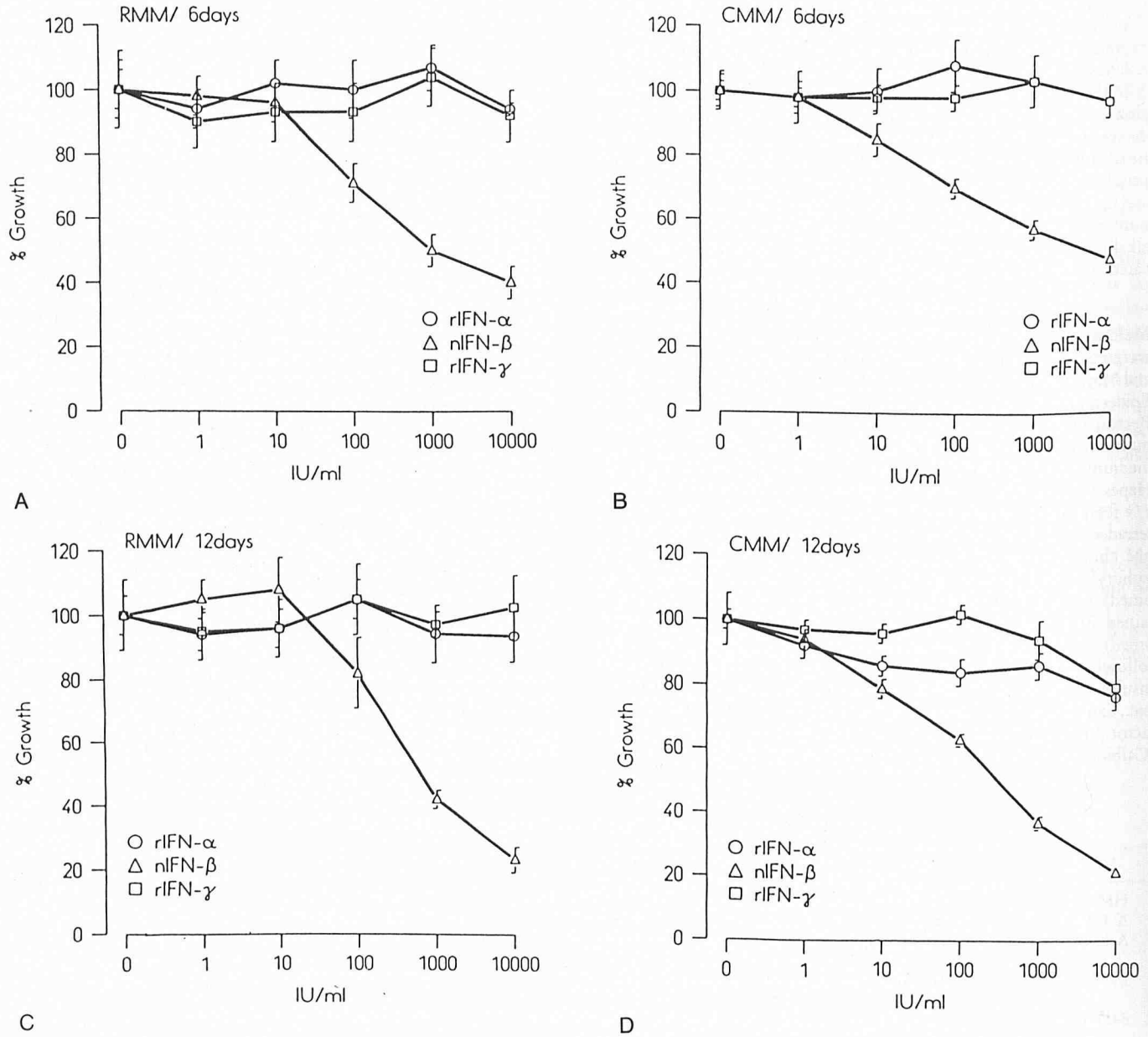

Figure 2. Antiproliferative effects of interferons on normal human melanocytes cultured in RMM or CMM; strong growth inhibition by nIFN-β in either RMM or CMM in both 6- and 12-d assays, slight inhibition by rIFN-α and rIFN-γ only in RMM in the 12-d assay. Representative results from one of three experiments.

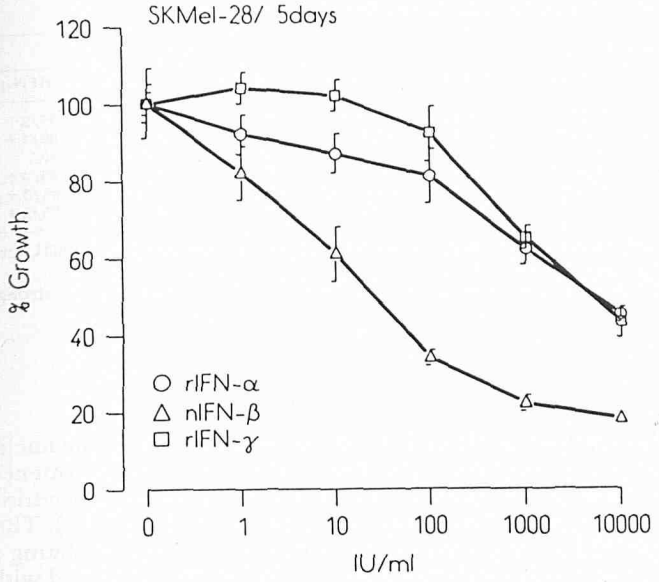

Figure 3. Effect of interferons on proliferation of melanoma cell line SKMel-28 under serum-free conditions; strong growth inhibition was obtained by all three interferons after 5 d. Representative results from one of three experiments.

K.1.2. as a marker of differentiated melanocytes [26], the A.1.43 reacting with a surface antigen as a melanoma cell progression marker [27], the MoAb B.9.12.1 for detecting HLA class I antigens [28], the MoAb L243 against the HLA-DR antigen [29], and the MoAb 84H10 reacting with the ICAM-1 antigen [30]. For further details see Table I. The visualization of the bound primary MoAb was revealed by incubation with a rabbit–anti-mouse IgG (Dakopatts, Glostrup, Denmark), followed by an antibody complex with APAAP (Dianova, Hamburg, FRG). The procedure was repeated once with a 10-min incubation period. The preparations were counterstained with Meyer's hematoxylin (1%). Negative controls were obtained by omitting the primary antibody.

Fluorescence Staining for Flow Cytometry (FACS-analysis) FACS-analysis compared to APAAP, is an accurate technique that presents well the cell surface antigens. These antigens may be trypsin sensitive. Pilot experiments have shown that ethylenediamine tetraacetic acid (EDTA) treatment (0.5% in PBS) of the cells for detachment was superior to standard trypsin treatment in the preservation of surface antigens and, therefore, has been preferred. Melanocytes cultured in MGM were rinsed twice with PBS and then incubated with 100 μl dilutions (Table I) of the primary MoAb in PBS for 30 min on ice. In addition to APAAP immunocytochemistry, the cultured cells were stained with MoAb BL 6, which reacts with the CD1 antigen, a Langerhans cell marker [31]. The MoAb HMB-45 and K.1.2. were omitted because their epitopes are mainly cytoplasmatic. Incubation with the primary antibody was

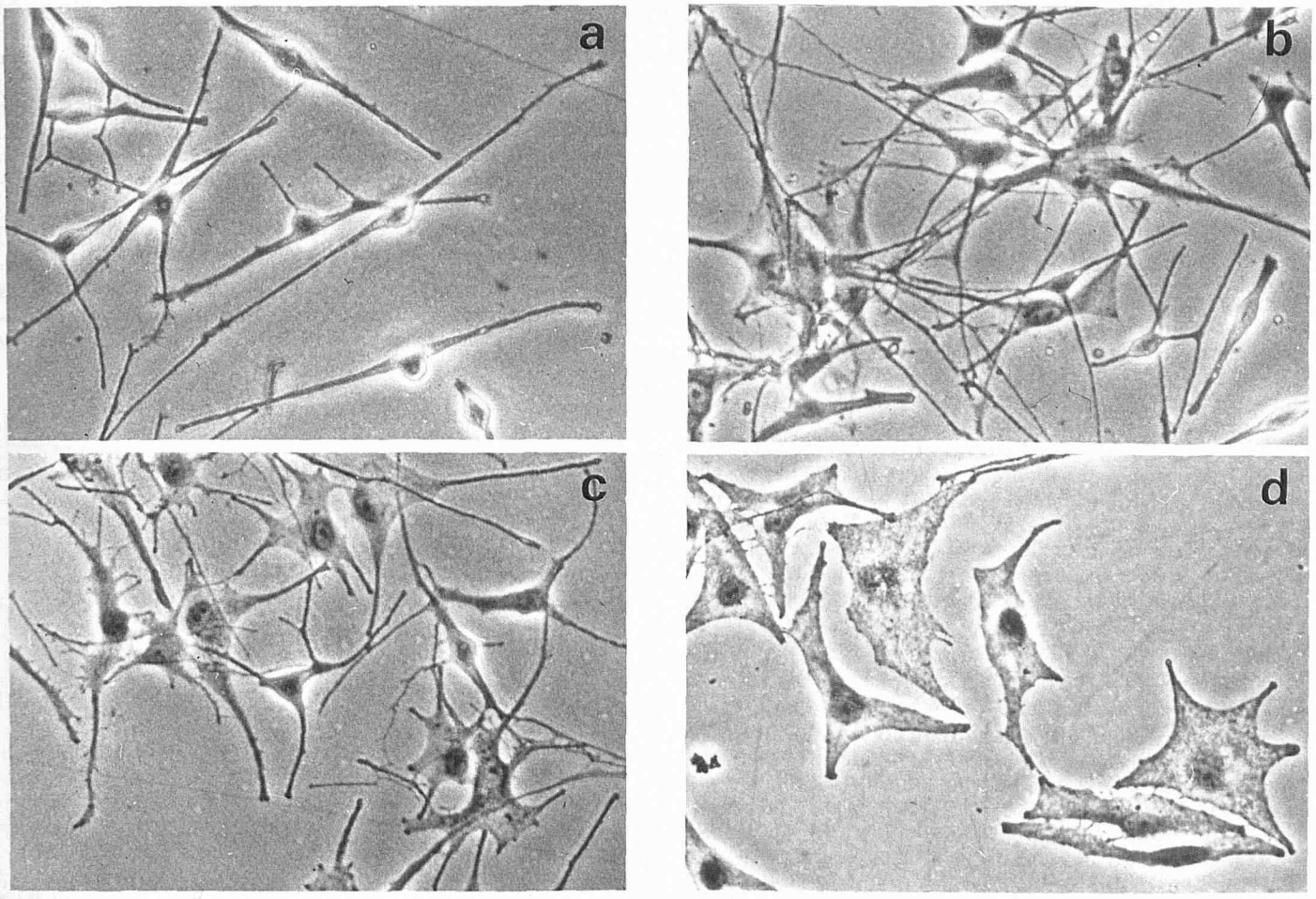

Figure 4. Newborn foreskin melanocytes exposed 5 d to 1000 IU/ml nIFN-β or rIFN-γ. Note increased dendricity of the nIFN-β–treated cultures *(b)* compared to the untreated controls *(a)*, and some cell flattening in rIFN-γ–treated cultures *(c)*. The effect of rIFN-γ was more profound on resting melanocytes *(d)* (magnification ×1200).

Table II. Antigenic Profile of Cultured Human Melanocytes and Modulation by Interferons In Vitro[a]

MoAb (Antigens)	Donor 1				Donor 2				Donor 3			
	Controls	rIFN-α	nIFN-β	rIFN-γ	Controls	rIFN-α	nIFN-β	rIFN-γ	Controls	rIFN-α	nIFN-β	rIFN-γ
HMB-45	95/3+[b]	NC[d]	95/2+	75/1+	100/4+	NC	95/2+	100/2+	100/4+	100/4+	90/2+	95/2+
K.1.2	40/2+	NC	30/1+	25/1+	95/3+	NC	80/1+	75/1+	95/3+	90/2+	65/1+	80/1+
A.1.43	5/2+	NC	NC	15/2+	5/2+	NC	NC	NC	5/2+	NC	15/2+	NC
HLA-I	95/3+	95/4+	95/4+	95/4+	100/3+	100/4+	100/4+	100/4+	95/3+	95/4+	95/4+	95/4+
HLA-DR	Neg.[c]	NC	15/2+	65/3+	Neg.	NC	15/2+	95/3+	Neg.	NC	20/2+	95/3+
ICAM-1	30/1+	NC	70/2+	90/3+	45/1+	NC	75/2+	95/3+	75/1+	NC	90/2+	95/3+

[a] Second passage foreskin melanocytes were plated at 10^4 cells/cm² in 35-mm culture dishes. At days 1 and 4 after seeding cultures were fed with media containing 0 or 1000 IU/ml of the various IFN. Antigen-positive cells and staining intensity were evaluated on day 6 after staining with APAAP.

[b] First number shows percentage of antigen-positive cells per culture; crosses (+) indicate intensity of staining reaction as follows: 1+ = weak; 2+ = moderate; 3+ = strong; 4+ = very strong.

[c] Neg. = negative.

[d] NC = no change.

followed by two washings with PBS and subsequent incubation of the living cells with 150 μl of a 1:40 dilution of fluorescein isothiocyanate (FITC) conjugated goat-anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) for 30 min on ice. Then the cells were washed three times with PBS and were finally suspended in PBS containing 1% paraformaldehyde. The fluorescence measurements were performed with a flow cytometer (Epics 752, Coulter Electronics) using a 488-nm argon laser beam. Non-specific staining was determined by omitting the first antibody.

Statistics Proliferation experiments were performed five to eight times; mean values and standard deviations were calculated. Statistical differences of the data were evaluated by the two-sided Student t test.

RESULTS

Cell Proliferation Proliferation in MGM is shown in Fig 1; rIFN-α at concentrations between 10 and 10,000 IU/ml resulted in a statistically significant but not dose-dependent increase of melanocytic proliferation as compared to untreated controls. Maximal stimulation occurred at 10 IU/ml with 140% growth of the controls ($p < 0.01$). Cultures incubated with low doses of rIFN-γ (1–10 IU/ml) also demonstrated slight stimulation, up to 130% of the controls at 1 IU/ml ($p < 0.01$). The rIFN-γ at concentrations ≥ 100 IU/ml and nIFN-β at 1–10,000 IU/ml did not alter the growth of cultured human melanocytes. Proliferation in CMM and RMM is shown in Fig 2; as MGM is a "supercharged" medium optimized for cell proliferation that may mask effects of interferons, experiments were also performed in CMM and RMM. nIFN-β dose dependently inhibited melanocyte proliferation in both CMM and RMM in a 6-d assay (10,000 IU/ml: 53% of controls in CMM and 61% of controls in RMM, $p < 0.001$, see also Fig 2). In the 12-d assay (Fig 2) stronger inhibition was observed (10,000 IU/ml: 80% of controls in CMM and 77% of controls in RMM, $p < 0.001$). With higher doses of nIFN-β (1,000–10,000 IU/ml) significant cytotoxicity was observed. In contrast to nIFN-β, rIFN-α and rIFN-γ showed no effect on melanocyte proliferation in RMM over a period of 12 d in culture (Fig 2). In CMM, a slight inhibitory effect with rIFN-α and rIFN-γ was seen in 12-d but not in the 6-d assay. Maximal inhibition was 24% of the controls with 10,000 IU/ml rIFN-α and 21% in the same concentration levels of rIFN-γ (both $p < 0.01$).

Effect of Interferons on Melanoma Cell Proliferation All three IFN dose dependently inhibited the proliferation of SKMel-28 melanoma cell line in a 5-d assay in serum-free medium (Fig 3). The strongest effect was carried by nIFN-β (78% of the controls with 10,000 IU/ml, $p < 0.001$). For rIFN-α and rIFN-γ, maximal effects were obtained also with 10,000 IU/ml, reaching 59% and 56% of the controls respectively (both $p < 0.001$).

Cell Morphology The morphology of cultured human melanocytes was clearly altered after 2–4 d treatment with 10 IU/ml rIFN-γ. Figure 4*a* shows the characteristic bipolar to dendritic morphology of neonatal foreskin melanocytes with predominant nuclei and scarce cytoplasm. After rIFN-γ, the cells presented flattened nuclei with well-spread cytoplasm and developed small dendritic processes, resembling melanocytes deprived of TPA (Fig 4*c*). This effect was more pronounced on resting melanocytes, exhibiting a tripolar to polygonal pattern (Fig 4*d*). Melanocytes incubated with nIFN-β were larger and developed multiple branched dendrites (Fig 4*b*). Only minor morphologic changes could be observed in rIFN-α treated melanocytes.

Antigenic Profile of Cultured Human Melanocytes In Vitro As revealed by the APAAP technique (Table II), nearly all cultured melanocytes grown in MGM showed strong granular cytoplasmic staining with the pigment cell-associated MoAb HMB-45. Depending on the donor, human melanocytes in culture revealed moderate (40%) to strong reactivity (100%) with the MoAb K.1.2; the K.1.2 antigen was localized mainly in the cytoplasmic area around the nucleus, long dendrites were only faintly or not stained at all (Fig 5). Investigations with both the APAAP-method and FACS-analysis (Fig 6) showed that only a small portion of the cells (<5%) were positive for the melanoma cell progression marker A.1.43. Both methods confirmed that most cultured melanocytes expressed HLA class I antigens, but none of them HLA-DR. Positive staining for ICAM-1 differed between the donors. No $CD1^+$ cells could be detected as was shown by MoAb BL 6.

Changes of the Antigenic Profile of Cultured Melanocytes after Treatment with Interferons Immunophenotypic examination of the melanocytes for modulation of antigen expression by

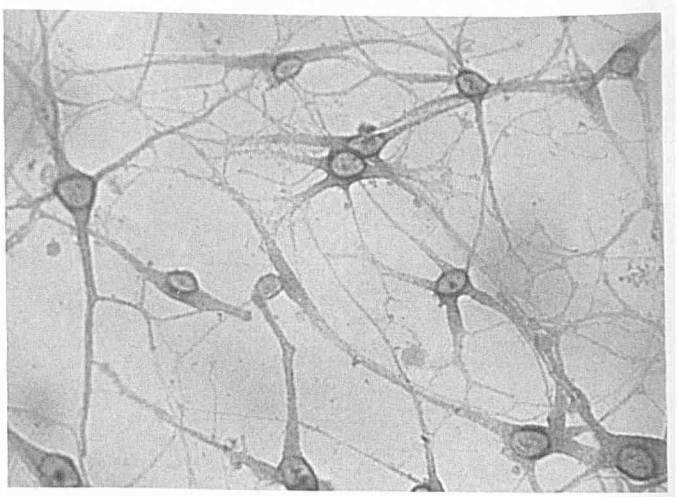

Figure 5. K.1.2-defined antigen expression on cultured melanocytes in the presence of TPA. Cells were grown in Heraeus Flexiperm chambers and were stained in situ with the APAAP technique. Note the strong perinuclear labeling and the weak or no staining of the dendrites (magnification ×1000).

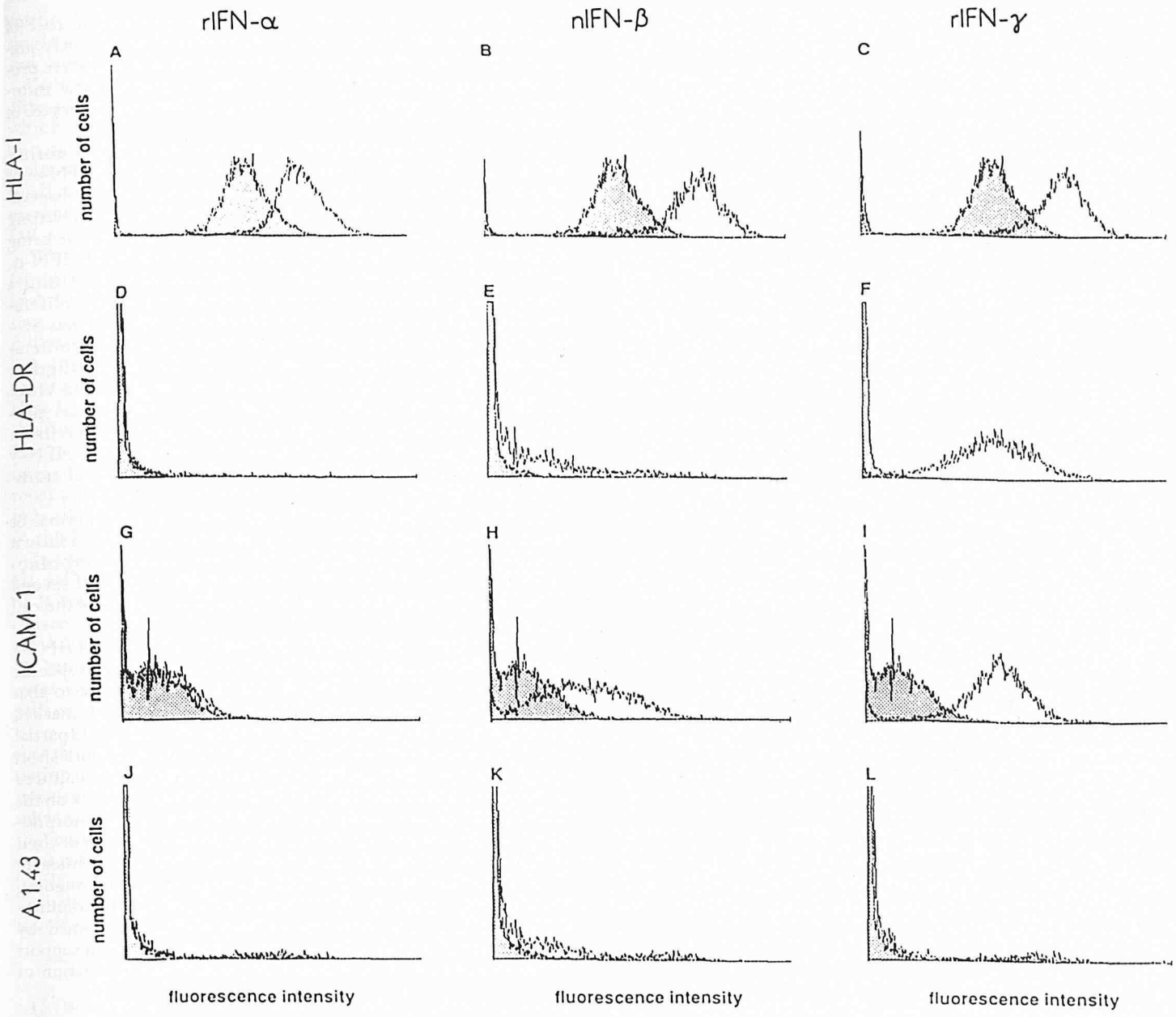

Figure 6. Induction of cell surface antigens on normal human melanocytes (donor 3) in vitro by 1000 IU/ml rIFN-α, nIFN-β, and rIFN-γ as found by FACS-analysis. Shaded figures represent histograms of the untreated control cultures. Enhancement of HLA-I expression under rIFN-α *(A)*, nIFN-β *(B)*, and rIFN-γ *(C)*, de novo induction of HLA-DR by nIFN-β *(E)* and rIFN-γ *(F)*, no change by rIFN-α *(D)*. Also, enhancement of the expression of the ICAM-1 molecule was clearly found by nIFN-β *(H)* and rIFN-γ *(I)*; rIFN-α *(G)* only slightly upregulated the ICAM-1 molecule. Upregulation of the A.1.43-defined antigen by nIFN-β *(K)*, rIFN-α *(J)* and rIFN-γ *(L)* did not change A.1.43 expression.

IFN was performed in MGM. The rIFN-α induced minor changes in the expression of antigens by cultured human melanocytes. The only clear change observed was a stronger expression of HLA class I antigens. In one of the melanocyte cultures treated with rIFN-α, a slight decrease of the K.1.2 antigen was seen.

Although sharing the same receptor with rIFN-α, nIFN-β clearly decreased the reaction with HMB-45 and K.1.2 (Table II). The progression marker A.1.43 was found upregulated after nIFN-β treatment only in one of the three cultures tested. The nIFN-β carried the strongest effect from all interferons regarding the upregulation of HLA class I antigens, as revealed by FACS-analysis (Fig 6). Interestingly enough, nIFN-β induced de novo expression of HLA-DR in $\leq 20\%$ of the cultured cells. The expression of the ICAM-1 antigen was also found increased, but to a lesser extent compared to that observed by rIFN-γ.

More markedly than both rIFN-α and nIFN-β, rIFN-γ modulated the antigenic phenotype of normal melanocytes in vitro. Reduction in the expression of the HMB-45 defined antigen was seen in all experiments after rIFN-γ treatment ($10^2 - 10^3$ IU/ml) and also after nIFN-β treatment ($10^2 - 10^3$ IU/ml). This effect was dose dependent. Cultured human melanocytes exposed to rIFN-γ exhibited decreased staining with MoAb K.1.2 (Fig 7). Changes in the expression of A.1.43 were found only in one of the three cultures tested (5–15%). The expression of HLA class I antigens was found elevated on rIFN-γ–treated melanocytes. Cells incubated with rIFN-γ at various concentration levels were found HLA-DR–positive dose dependently up to 10^3 IU/ml; at higher doses, decreased induction of HLA-DR was observed (Fig 8). The percentage of HLA-DR–positive cells after rIFN-γ varied between 65% and 95%. Dose-dependent increment in the percentage of ICAM-1–positive cultured melanocytes was observed with $10^2 - 10^3$ IU/ml rIFN-γ to maximal 95%. For the described changes of immuno-

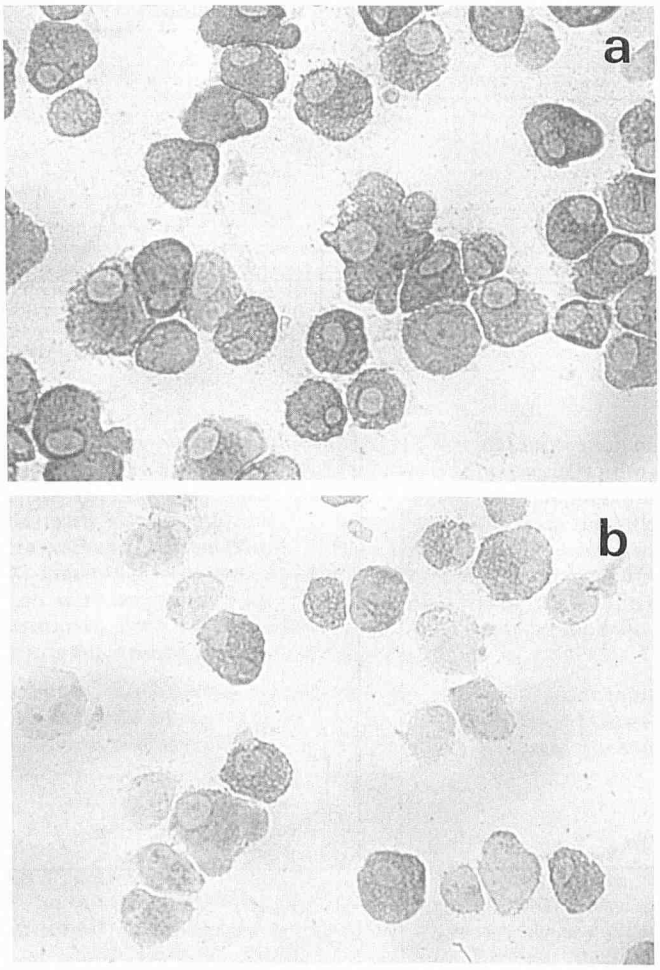

Figure 7. rIFN-γ decreases the expression of the K.1.2-defined antigen. Cytospin preparations of untreated *(a)* and with 1000 IU/ml rIFN-γ–treated melanocytes of donor 2 *(b)* were simultaneously stained with 1:2 dilution of the K.1.2 MoAb. The intensity of cell labeling and the number of the antigen-positive cells were substantially decreased in rIFN-γ–treated cultures (magnification $\times$1000).

phenotype induced by interferons, dose-related augmentation of staining intensity was observed. Exceptions were the downregulation of K.1.2 by rIFN-α, the induction of A.1.43 by nIFN-β and rIFN-γ as well as of HLA-DR by nIFN-β.

DISCUSSION

Experiments performed in MGM for assessment of antiproliferative action of interferons on normal melanocyte cultures revealed no significant growth inhibition. As this medium contains high concentrations of TPA and fetal calf serum that might "superstimulate" cultured melanocytes, experiments were also performed in a TPA- and serum-free but complete melanocyte medium (CMM) and in a mitogen-reduced variant (RMM) that contained no transferrin, bFGF or cholera toxin. In CMM, foreskin melanocytes show a rapid proliferation (35–70% positivity for Ki-67 antigen, data not shown), whereas in RMM less or no proliferation occurs. The findings in CMM and RMM revealed that nIFN-β strongly inhibits the proliferation of skin-derived normal human melanocytes in vitro in a dose-dependent manner; higher doses of nIFN-β (1,000–10,000 IU/ml) were cytotoxic. In contrast to nIFN-β, in CMM exerted rIFN-α and rIFN-γ only marginal antiproliferative effects on melanocytic growth. This effect could not be demonstrated in CMM in the 6-d assay, and not at all in RMM. The physiologic role of nIFN-β in inhibiting melanocyte proliferation is presently unknown. Possibly, nIFN-β is a negative regulator of melanocyte proliferation suppressing melanocyte response to keratinocytic mitogens (i.e., bFGF or α-MSH) and keeping epidermal melanocytes in senescence.

From our present findings it is apparent that cultured normal melanocytes show little or no growth response to either rIFN-α or rIFN-γ. These results are in sharp contrast to our earlier observations on melanoma cell lines, where all three IFN exerted strong antiproliferative effects in 3-d assays. In the present article, we bring additional evidence for the growth inhibitory effects of rIFN-α, nIFN-β and of rIFN-γ on the SKMel-28 melanoma cell line using a 5-d serum-free assay. In this system maximal inhibition of proliferation of SKMel-28 melanoma cell line with different IFN was 55–82%. It seems that rIFN-α and rIFN-γ exert increased antiproliferative activity on transformed melanocytes and that malignant transformation evokes increased sensitivity to interferons in vitro. In this respect, the pathway of growth regulation appears profoundly changed between normal and transformed pigment cells. In other epidermal cell populations, i.e., in keratinocytes, rIFN-γ strongly inhibits the proliferation of both normal and of transformed cells [32,33].

The exact mechanisms by which interferons suppress normal or tumor cell proliferation is not clearly understood. It has been shown that IFN may act as negative growth factors by antagonizing mitogenic agents [34]. They decrease the intracellular level of several known oncogens [35,36] and cause significant alterations of the cell cycle [37].

The decreased antiproliferative activity of rIFN-α and of rIFN-γ on normal human melanocytes is not due to the absence of specific IFN receptors, as IFN modulated their phenotype similar to that reported for melanoma cells [13]. The rIFN-γ caused marked changes of melanocytic morphology with cell flattening and partial loss of the bipolar dendrites. Thus, epitheloid-like cells with short cytoplasmic processes appeared, showing similarities to cultured melanoma cells. A direct action of the IFN-receptor complex on the organization of the cytoskeleton may contribute to this morphologic change. Possibly, the induction of adhesion proteins or their receptors, i.e., of fibronectin and other members of the integrin family, modulate melanocyte adhesion and spreading. Still ongoing investigations suggest that rIFN-γ increases the amount of immunoreactive cellular fibronectin on melanocytes, as determined by MoAb specific for human fibronectin (data not shown). In support of our observations, Varani et al [38] reported that incubation of

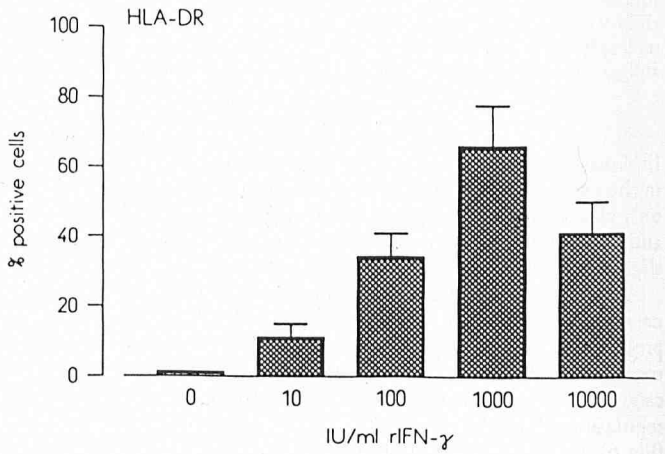

Figure 8. Cultured melanocytes of donor 1 were treated 5 d with rIFN-γ and HLA-DR+ cells were counted after staining of cytospin preparations with L243 MoAb using the APAAP technique. Maximal induction was obtained with 1000 IU/ml rIFN-γ (n = 3).

cultured melanocytes with rIFN-γ and TNF-α, but not with the single substances for 1 d resulted in increased fibronectin production and morphologic changes similar to those described here.

Cultured human melanocytes presented the antigenic profile of differentiated pigment cells with the expression of K.1.2 antigen, which declines with tumor progression [15,26], and weak expression of the progression markers A.1.43 and ICAM-1 [15]. Cultured melanoma cells expressed the differentiation marker K.1.2 only to a small degree whereas they were strong positive for the progression marker A.1.43 [13]. On the other hand, they did not express HLA-DR antigens.

We found that nIFN-β and especially rIFN-γ induced a less differentiated phenotype on cultured melanocytes, and that nIFN-β and rIFN-γ both decreased K.1.2 expression, enhanced the expression of ICAM-1 and induced de novo expression of HLA-DR. Additionally, we found increased expression of the progression marker A.1.43 during nIFN-β or rIFN-γ treatment. In contrast to previous reports that only 40–80% of normal melanocytes in vitro are HLA class I positive [17], our investigations revealed that 95–100% of the human melanocytes cultured in vitro expressed high levels of HLA class I antigens, and that these antigens were prone to further upregulation by all three interferons tested, with nIFN-β being the most potent inducer. Possibly, some differences between our findings and those from other authors are due to the different sensitivity of the MoAb as well as of the methods used for evaluation.

In addition to rIFN-γ, nIFN-β also induced de novo expression of HLA-DR on cultured human melanocytes, as well as augmentation of ICAM-1 but this effect was less pronounced compared to that caused by rIFN-γ. Similar observations regarding the induction of HLA-DR by nIFN-β were reported for cultured melanoma cells.

Our findings on antigen modulation of cultured normal melanocytes induced by IFN are similar to those reported for melanoma cells and indicate that IFN, especially rIFN-γ, may generally induce a less differentiated cell phenotype in vitro. Furthermore, it seems that the regulation of melanocytic proliferation, but not of differentiation by rIFN-α and rIFN-γ, changes in vitro with their malignant transformation. Together with their antiproliferative effects, to what extent these alterations of the antigenic phenotype account for the antitumor activities of the IFN remains unclear.The predominant induction of HLA class I and II antigens, as well as of ICAM-1, by rIFN-γ corresponds to similar findings recently reported on endotheliocytes and other cell models [32,39,40] and may suggest involvement of human melanocytes in immunologic skin processes.

REFERENCES

1. Worman CP, Catovsky D, Beran PC, Camba L, Joyner M, Green PJ, Williams HJH, Boomley JM, Gordon-Smith EC, Cawley JC: Interferon is effective in hairy cell leukemia. Br J Hamatol 60:759–763, 1985
2. Foon KA, Sherwin SA, Abrams PG, Longo DL, Fer MF, Stevenson HC, Jeffrey JO, Bottino GC, Schoenberger CS, Zeffren J, Jaffe ES, Oldam RK: Treatment of advanced non Hodgkin's lymphoma with recombinant leukocyte A interferon. N Engl J Med 311:1148–1152, 1985
3. Schaart FM, Bratzke B, Stadler R, Orfanos CE: HIV-associated Kaposi's sarcoma responds to long-term alpha interferon (rIFN alpha 2a) monotherapy (abstr). J Invest Dermatol 93:576, 1989
4. Clemens MJ, McNurlan MA: Regulation of cell proliferation and differentiation by interferons. Biochem J 226:345–360, 1985
5. Fischer PB, Grant S: Effects of interferon on differentiation of normal and tumor cells. Pharmacol Ther 27:143–166, 1985
6. Houghton AN, Thomson TM, Gross D, Oettgen HF, Old LJ: Surface antigens of melanoma and melanocytes: specificity of induction of Ia antigens by human γ-interferon. J Exp Med 160:255–269, 1984
7. Capobianchi MR, Ameglio F, Tosi R, Dolei A: Differences in the expression and release of DR, BR, and DQ molecules in human cells treated with recombinant interferon gamma: comparison to other interferons. Hum Immunol 13:1–11, 1985
8. McLeod GR: Clinical evaluation of interferon therapy in malignant melanoma (abstr). J Invest Dermatol 93:563, 1989
9. Kokoschka EM, Trautinger F, Miscksche M, Kokoschka R: Long-term adjuvant therapy of high-risk malignant melanoma with recombinant interferon alpha 2b (abstr). J Invest Dermatol 93:560, 1989
10. Czarniecki CW, Fennie CW, Powers DB, Estell DA: Synergistic antiviral and antiproliferative activities of escherichia coli derived human alpha, beta and gamma interferons. J Virol 49:490–496, 1984
11. Liao S-K, Kwong PC, Khosravi M, Dent PB: Enhanced expression of melanoma-associated antigens and β_2-microglobulin on cultured human melanoma cells by interferon. J Natl Cancer Inst 68:19–25, 1982
12. Carrel S, Schmidt-Kessen A, Giuffre L: Recombinant interferon-gamma can induce the expression of HLA-DR and DC on DR negative melanoma cells and enhance the expression of HLA ABC and tumor associated antigens. Eur J Immunol 15:118–124, 1985
13. Garbe C, Krasagakis K, Zouboulis ChC, Schröder K, Krüger S, Stadler R, Orfanos CE: Antitumor activities of interferon-alpha, -beta and -gamma and their combinations on human melanoma cells in vitro: changes of proliferation, melanin synthesis and immunophenotype. J Invest Dermatol 95:231S–237S, 1990
14. Bröcker EB, Suter L, Brüggen J, Ruiter DJ, Macher E, Sorg C: Phenotypic dynamics of tumor progression in human malignant melanoma. Int J Cancer 36:29–35, 1985
15. Holzmann B, Bröcker EB, Lehmann JM, Ruiter DJ, Sorg C, Riethmüller G, Johnson JP: Tumor progression in human malignant melanoma: five stages defined by their antigenic phenotypes. Int J Cancer 39:466–471, 1987
16. Herlyn M, Guerry D, Koprowski H: Recombinant γ-interferon induces changes in expression and shedding of antigens associated with normal human melanocytes, nevus cells, and primary and metastatic melanoma cells. J Immunol 134:4226–4230, 1985
17. Tsujisaki M, Igarashi M, Sakaguchi K, Eisinger M, Herlyn M, Ferrone S: Immunochemical and functional analysis of HLA class II antigens induced by recombinant immune interferon on normal epidermal melanocytes. J Immunol 138:1310–1316, 1987
18. Temponi M, Romano G, D'Urso CM, Wang Z, Kekish U, Ferrone S: Profile of intercellular adhesion molecule-1 (ICAM-1) synthesized by human melanoma cell lines. Semin Oncol 15:595–607, 1988
19. Eisinger M, Marco O: Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci USA 79:2018–2022, 1982
20. Gilchrest BA, Vrabel MA, Flynn E, Szabo G: Selective cultivation of human melanocytes from newborn and adult epidermis. J Invest Dermatol 83:370–376, 1984
21. Pittelkow MR: New techniques for the in vitro culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. Mayo Clin Proc 61:771–777, 1986
22. Carey TE, Takahashi T, Resnick LA, Oettgen HF, Old LJ: Cell surface antigens of human malignant melanoma: mixed hemadsorption assays for humoral immunity to cultured autologous melanoma cells. Proc Natl Acad Sci USA 73:3278–3282, 1976
23. Stadler R, Detmar M, Stephanek K, Bangemann C, Orfanos CE: A rapid fluorometric assay for the determination of keratinocyte proliferation in vitro. J Invest Dermatol 93:532–534, 1989
24. Garbe C, Krüger S, Zouboulis ChC, Krasagakis K, Schröder K, Orfanos CE: Determination of antiproliferative drug effects on human malignant melanoma cells in vitro by a rapid fluorometric microassay (abstr). Arch Dermatol Res 281:585, 1989
25. Gown AM, Vogel AM, Hoak D, Gough F, McNutt MA: Monoclonal antibodies specific for melanocytic tumors distinguish subpopulations of melanocytes. Am J Pathol 123:195–203, 1986
26. Suter L, Brüggen J, Bröcker EB, Sorg C: A tumor-associated antigen expressed in melanoma cells with lower malignant potential. Int J Cancer 35:787–791, 1985
27. Brüggen J, Sorg C: Detection of phenotypic differences on human malignant melanoma lines and their variant sublines with monoclonal antibodies. Cancer Immunol Immunother 15:200–205, 1983
28. Malissen B, Rebai N, Liabeuf A, Mawas C: Human cytotoxic T-cell structures associated with expression of cytolysis. I. Analysis at the clonal level of the cytolysis-inhibiting effect of 7 monoclonal antibodies. Eur J Immunol 12:739–747, 1982
29. Lampson LA, Levy R: The populations of Ia-like molecules on a human B-cell line. J Immunol 125:293–299, 1980

30. Rothlein R, Dustin MC, Marlin SD, Springer TA: A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol 137:1270–1275, 1986

31. Yonish-Rouach E, Viak J, Schmitt D, Cordier G, Thivolet J, Brochier J: Preparation of monoclonal antihuman thymocyte antibodies: unexpected markers of epidermal cells. In: Serrou B, et al (eds.). Current concepts in Human Immunology and Cancer Immunotherapy. Elsevier Biomedical Press, New York, 1982, pp 17–55

32. Symington FW: Lymphotoxin, tumor necrosis factor, and gamma interferon are cytostatic for normal human keratinocytes. J Invest Dermatol 92:798–805, 1989

33. Bangemann C, Stadler R: Antitumor effects of natural and recombinant interferons on transformed human keratinocytes (abstr). J Invest Dermatol 93:539, 1989

34. Inglot AD, Pajtasz E: Measurement of antagonistic effects of growth factors and interferons. Methods Enzymol 119:657–667, 1986

35. Dani C, Mechti N, Piechaczyk M, Lebleu B, Jeanteur P, Blanchard JM: Increased rate of degradation of c-myc in RNA of interferon-treated Daudi cells. Proc Natl Acad Sci USA 82:4896–4899, 1985

36. Samid D, Chang EH, Friedman RE: Biochemical correlates of phenotypic reversion in interferon-treated mouse cells transformed by a human oncogene. Biochem Biophys Res Commun 119:21–28, 1984

37. Creasy AA, Bartholomew JC, Merigan TC: Role of G_o-G_1 arrest in the inhibition of tumor cell growth by interferons. Proc Natl Acad Sci USA 77:1471–1475, 1980

38. Varani J, Mitra RS, McClenic BJ, Fligiel SEG, Inmann DR, Dixit VM, Nickoloff BJ: Modulation of fibronectin production in normal melanocytes and malignant melanoma cells by interferon-γ and tumor necrosis factor-α. Am J Pathol 134:827–836, 1989

39. Ruszczak ZB, Detmar M, Imcke E, Orfanos CE: Effects of rIFN-alpha, rIFN-beta and rIFN-gamma on the morphology, proliferation and cell surface antigen expression on human dermal microvascular endothelial cells in vitro. J Invest Dermatol 95:693–699, 1990

40. Dustin ML, Singer KH, Tuck DT, Springer TA: Adhesion of T-lymphoblasts to epidermal keratinocytes is regulated by interferon gamma and is mediated by intercellular adhesion molecule-1 (ICAM-1). J Exp Med 167:1323–1340, 1988